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Recombinant Infectious Bronchitis Viruses expressing chimaeric spike glycoproteins induce partial protective immunity against homologous challenge despite limited replication *in vivo*

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Running title: rIBV expressing full S partially protects against IBV

Keywords: Avian Infectious bronchitis virus, coronavirus, spike, S1, protection, recombinant vaccine, BeauR

Word counts: manuscript text (5989 words); abstract (248 words); importance (147 words)

ABSTRACT

Vaccination regimes against *Infectious bronchitis virus*, which are based on a single virus serotype, often induce insufficient levels of cross-protection against serotypes and two or more antigenically diverse vaccines are used in attempt to provide broader protection. Amino acid differences in the surface protein, spike (S), in particular the S1 subunit, are associated with poor cross-protection. Here, homologous vaccination trials with recombinant IBVs, based on the apathogenic strain, BeauR, were conducted to elucidate the role of S1 in protection. A single vaccination of SPF-chickens with rIBV expressing S1 of virulent strains M41 or QX, BeauR-M41(S1) and BeauR-QX(S1), gave incomplete protection against homologous challenge, based on ciliary activity and clinical signs. There could be conformational issues with the spike if heterologous S1 and S2 are linked, suggesting a homologous S2 might be essential. To address this, a homologous vaccination-challenge trial incorporating rIBVs expressing full spike from M41, BeauR-M41(S), and S2 subunit from M41, BeauR-M41(S2) was conducted. All chimaeric viruses grew to similar titres *in vitro*, induced virus-specific partial protective immunity, evident by cellular infiltrations, reductions in viral RNA load in the trachea and conjunctiva and higher serum anti-IBV titres. Collectively, these show that vaccination with rIBVs primed the birds for challenge but the viruses were cleared rapidly from the mucosal tissues in the head. Chimaeric S1 and S2 viruses did not protect as effectively as BeauR-M41(S) based on ciliary activity and clinical signs. Booster vaccinations and a rIBV with improved *in vivo* replication may improve the levels of protection.

IMPORTANCE

Infectious bronchitis virus causes an acute, highly contagious respiratory disease, responsible for significant economic losses to the poultry industry. Amino acid differences in the surface protein, spike (S), in particular the S1 subunit, have been associated with poor cross-protection. Available vaccines give poor cross-protection and rationally designed live attenuated vaccines, based on apathogenic BeauR, could address these. Here, to determine the role of S1 in protection, a series of homologous vaccination trials with rIBVs were conducted. Single vaccinations with chimaeric rIBVs induced virus-specific partial protective immunity, characterised by reduction in viral load and serum antibody titres. However, BeauR-M41(S) was the only vaccination to improve the level of protection against clinical signs and the loss of tracheal ciliary activity. Growth characteristics show all of the rIBVs replicated *in vitro* to similar levels. Booster vaccinations and a rIBV with improved *in vivo* replication may improve the levels of protection.

INTRODUCTION

Infectious Bronchitis virus (IBV) is classified as a *gammacoronavirus*, subfamily *Coronavirinae*, order *Nidovirales* (1). IBV is responsible for major economic losses to poultry industries worldwide as a result of poor weight gain, decreased egg production and impaired egg quality. The effect of IBV on the ciliary activity in the trachea and the immune system may predispose infected chickens to secondary infections with opportunistic bacteria, which often increases the mortality rate associated with IBV (2-4).

IBV is an enveloped virus, with a single-stranded, positive sense RNA genome (~28kB), and encodes four structural proteins: nucleocapsid (N), spike glycoprotein (S), small membrane protein

envelope (E) and integral membrane protein (M) (5, 6). The major surface protein of IBV, S, is a type 1 glycoprotein which oligomerises to form trimers (7) and is thought to be the main inducer of protective immunity (8-12). The S protein is proteolytically cleaved into two subunits, the N-terminal subunit S1 (approx. 500-550 amino acids, 90-kDa) and the C-terminal subunit, S2 (630 amino acids, 84-kDa), which contains the transmembrane domain. The S1 subunit plays a critical role in binding to cellular receptors as it contains the receptor binding domain (13, 14), determines the virus serotype and is responsible for the induction of neutralising antibodies (14-16). Multiple studies have shown that recombinant S1 expressed in adenovirus and Newcastle Disease Virus vectors can induce a certain level of protection in specified-pathogen free (SPF) chickens against challenge with wild-type virus (11, 17, 18).

Vaccine programmes against IBV often include a combination of live or inactivated vaccines which are based on several dominant field serotypes of the virus. The current vaccines often induce insufficient cross-protection, and combinations of antigenically different vaccines are used in an effort to improve levels of protection (19). Alongside this, with the continual emergence of new field strains the control of IBV is persistently a significant problem to the poultry industry.

A reverse genetics system based on the avirulent strain of IBV Beaudette has been developed (20, 21). This system has many potential applications, including; to enhance our understanding of the role of individual genes in pathogenicity and to lead to a new generation of rationally designed live attenuated vaccines (20). Previous work using the reverse genetics approach demonstrated that replacement of the ectodomain of the S glycoprotein of the apathogenic IBV Beaudette strain with the same region from either of two pathogenic IBV strains, M41-CK or 4/91, resulted in two non-virulent rIBVs, BeauR-M41(S) and BeauR-4/91(S), respectively. Notably, both rIBVs based on the BeauR backbone acquired the same cell tropism of that of the donor S, M41-CK or 4/91 (22,

23). Other work demonstrated that the Beaudette S2 subunit confers the unique ability of Beaudette to replicate in African Green Monkey Kidney (Vero) cells, a continuous cell line licensed for vaccine production (24, 26). Vaccination with BeauR-M41(S) or BeauR-4/91(S) can confer protection against homologous challenge based on ciliary activity, reductions in clinical signs and viral load in the trachea at 5 days post-challenge (dpc), further demonstrating the dominant role of the S glycoprotein in inducing protective immunity (23, 25).

In this study we investigated the protection conferred against homologous challenge by two rIBVs, BeauR-M41(S1) and BeauR-QX(S1), that contain S1 subunits from economically relevant strains, M41 and QX respectively, with the S2 subunit derived from BeauR. Notably both rIBVs have the advantageous ability to replicate in Vero cells (26; Bickerton *et al*. submitted for publication) due to the presence of the Beaudette S2 subunit. We report here on the first application of rIBV with a chimaeric S gene to be used in a vaccination trial. The rIBV BeauR-M41(S2) was also investigated in order to elucidate the relevant roles of both subunits in protective immunity. Whilst the S1 subunit is considered to be immunodominant, the S2 subunit is highly conserved between strains and contains immunogenic regions (14, 27).

We have shown here that vaccination with a recombinant IBV expressing a chimaeric S gene can induce a partially protective response against challenge, as assessed by viral load, cellular infiltration, clinical signs and a boost in serum antibody titres post-challenge. Vaccination with rIBV expressing homologous S1 and S2 subunits (i.e. full S gene) in the Beaudette backbone induced partial protection classified by the level of ciliary activity and presence of clinical signs following challenge with wild-type IBV. Comparison of *in vitro* growth characteristics shows that inclusion of a foreign S gene or a chimaeric S gene in the rIBVs does not impede replication *in vitro*. However, our data show that despite the ability to induce a degree of virus-specific protective

immunity, the rIBVs are hindered by limited *in vivo* replication and the attenuated BeauR backbone.

RESULTS

Characterisation of rIBV BeauR-M41(S1) and BeauR-QX(S1) for homologous protection. To determine if a single vaccination with rIBV expressing the S1 subunit of the S gene (with a Beaudette derived S2 subunit) was sufficient to induce protection against challenge with homologous pathogenic isolates of IBV, a vaccination/challenge trial was conducted with BeauR-M41(S1) and BeauR-QX(S1). No clinical signs nor loss of ciliary activity in the trachea were observed in either of the vaccinated groups following vaccination (data not shown). These results showed that replacement of the BeauR S1 gene with the S1 gene from pathogenic strains did not confer pathogenicity to the resulting BeauR-M41(S1) and BeauR-QX(S1) viruses.

Three weeks after the primary inoculation, chickens were challenged with a homologous wild-type virus strain, M41-CK or QX. Clinical signs were at the highest level in the challenge control groups, with QX more pathogenic than M41-CK (Fig. 2A and 2B). The rIBV vaccines expressing the S1 subunit did not confer full protection against clinical signs associated with IBV, although snicking and rales in the group vaccinated with QX(S1) resolved quicker than the QX challenge control (Fig. 2A and 2B). Vaccination with BeauR-M41(S1) or BeauR-QX(S1) did not prevent the loss of ciliary activity in the trachea following challenge with the homologous wild-type virus (Table 1).

To investigate the tissue tropism of the rIBVs, a range of tissues collected at 2 and 4 days post-vaccination (dpv) were assessed by RT-PCR. BeauR-M41(S1) and BeauR-QX(S1) RNA was not

130 detected in the conjunctiva, Harderian gland, nasal-associated lymphoid tissue (NALT) or trachea
131 at 2 and 4 dpv (data not shown). Histological analysis of the head-associated lymphoid tissues
132 revealed cellular infiltrates in both the Harderian gland and the conjunctiva-associated lymphoid
133 tissue (CALT) at 2 dpv (Fig. 3A-3D), with areas of CALT more prominent in vaccinated tissues
134 compared to Mock (Fig. 3E). Collectively, these suggest that the recombinant vaccine viruses did
135 infect these tissues but were no longer detectable by PCR at 2 dpv, suggesting rapid clearance from
136 the sites of inoculation and mucosal tissues in the head-associated lymphoid tissues, exerted by a
137 virus-specific protective immune response.

138 To elucidate if BeauR-M41(S1) and BeauR-QX(S1) were able to confer a degree of protection
139 against homologous challenge, evident by a reduction in viral load of infected tissues post-
140 challenge, qPCR was conducted to assess the level of viral RNA in trachea and CALT. At 2 dpc,
141 IBV viral RNA load in both trachea and CALT were significantly lower in the BeauR-M41(S1)
142 vaccinated groups compared to challenge controls (Fig. 4A and 4C), but at 4 dpc the viral RNA
143 load was only significantly lower in the CALT of the BeauR-QX(S1) vaccinated group (Fig. 4B
144 and 4D). Infectious viral load determined by titration of trachea tissue supernatant in TOCs,
145 showed a reduction in infectious virions recovered from BeauR-M41(S1) and BeauR-QX(S1)
146 vaccinated chickens, although not significant compared to corresponding wild-type controls
147 (BeauR-M41(S1), P=0.961 and BeauR-QX(S1), P=0.999) (Fig. 4E). The wild-type control groups
148 were the only groups to report significantly higher infectious viral loads recovered from the trachea
149 compared to that of the Mock/Mock controls (Fig. 4E).

150 Serum IBV-specific antibodies were assessed post-vaccination (pre-challenge) at 21 dpv and at 2,
151 4 and 14 dpc. Compared to the challenge control group, titres were significantly higher in the
152 BeauR-QX(S1) vaccinated group at 2 and 4 dpc (Fig. 5A and 5B) (P<0.05 and P<0.01,

153 respectively). At 14 dpc, serum titres were higher in both the BeauR-M41(S1) and BeauR-QX(S1)
154 vaccinated groups compared to the challenge control groups, but only the QX vaccinated group
155 was significantly higher compared to the corresponding challenge control group (Fig. 5C and 5D)
156 (P<0.05). For both vaccinated groups, antibody titres at 21 dpv (pre-challenge) could be classed
157 as "borderline" positive due to being above the limits of the S/P cut-off (Fig. 5C and 5D).

158 In summary, results from Trial 1 suggest that although vaccination of the chickens with BeauR-
159 M41(S1) and BeauR-QX(S1) did not confer complete protection against homologous challenge
160 based on clinical signs and ciliary activity, a single vaccination of young chickens induced a
161 partially protective virus-specific immune response as indicated by a significant reduction in viral
162 load in trachea and CALT tissues. Higher IBV-specific serum antibody titres compared to
163 challenge-only controls shows that vaccination with chimaeric rIBVs were able to prime the birds
164 for challenge. Whether the lack of full protection against the loss of ciliary activity and clinical
165 signs was due to the absence of a homologous S2 subunit or an incorrect folding of M41/QX (S1)
166 and BeauR (S2) and therefore lower infectivity could not be answered in this specific study.
167 Therefore, a second trial addressing the issue of whether a homologous S2 is required for
168 protection was conducted.

169 **Relative contribution of S1 and S2 to homologous protection.** In Trial 2, the rIBV used were;
170 BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) (described in Fig. 1), with a similar
171 experimental design to that of Trial 1. No clinical signs were observed in any of the vaccinated
172 groups after vaccination (data not shown). Following vaccination, there was no loss of ciliary
173 activity in the trachea, indicating the apathogenicity of the rIBVs (data not shown). In the same
174 manner to Trial 1, at 21 dpv the chickens were challenged with M41-CK. Clinical signs were
175 observed until 7 dpc, BeauR-M41(S) was the only vaccinated group to show less prevalent clinical

176 signs post-challenge compared to the M41-CK challenge control (Fig. 6A and 6B). There was
177 little difference between the BeauR-M41(S1), BeauR-M41(S2) and M41-CK groups in terms of
178 the presence and severity of clinical signs (Fig. 6A and 6B), but in the vaccinated groups clinical
179 signs resolved more rapidly compared to the M41-CK controls. Ciliary activity was assessed at 4
180 dpc and the level of protection afforded were assessed according to European Pharmacopeia
181 standards (28). The BeauR-M41(S) vaccinated group retained ~60% ciliary activity, showing an
182 improved level of protection in comparison to groups vaccinated with BeauR-M41(S1) and
183 BeauR-M41(S2), in which 20% protection in each group were evident (Table 2). Noteworthy,
184 assessment on an individual bird level showed that 3 out of 5 birds in the BeauR-M41(S) were
185 classed as "protected against ciliostasis" however, as the group average was 60% this does not
186 translate into protection on a group level (Table 2).

187 Viral RNA loads in the tracheas and CALTs isolated from challenged chickens were determined
188 by qPCR to elucidate whether the S1 and S2 subunits played any further role in conferring
189 protection. At 2 dpc only the CALT from BeauR-M41(S) and BeauR-M41(S2) vaccinated
190 chickens showed any significant reduction ($P<0.001$) in viral RNA load compared to the challenge
191 control (Fig. 7A). However, at 4 dpc all groups had significantly lower viral RNA loads in the
192 CALT (Fig. 7B) ($P<0.001$, BeauR-M41(S) and $P<0.01$, BeauR-M41(S1) and BeauR-M41(S2)).
193 Viral RNA loads in the trachea were only significantly lower at 2 dpc in BeauR-M41(S) vaccinated
194 chickens ($P<0.001$, BeauR-M41(S)) and significantly lower for all vaccinated groups at 4 dpc (Fig.
195 7C and 7D) ($P<0.05$, BeauR-M41(S) and BeauR-M41(S2), $P<0.01$, BeauR-M41(S1)). Failure to
196 locate the rIBVs in the head-associated lymphoid and respiratory tissues at 2 dpv in Trial 1, lead
197 to the inclusion of the 1 dpv time-point in Trial 2. BeauR-M41(S), BeauR-M41(S1) and BeauR-
198 M41(S2) were detected by RT-PCR in a number of the Harderian glands and tracheas isolated

199 from chickens at 1 dpv, however, at 2 and 4 dpv the rIBVs were mainly detected in the nasal
200 turbinates (Table 3), suggesting rapid clearance of rIBVs from the mucosal head tissues and sites
201 of inoculation. Although, the titres of infectious challenge virus recovered from tracheas at 4 dpc
202 were not significantly reduced in BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2)
203 vaccinated chickens compared to controls (because of the variation within each group), there was
204 a general trend that vaccination resulted in a reduction in viral infectivity, with no detected
205 infectious virus recovered in 4 out of 5 birds in the (S) group, 3 out of 5 in the S1 group and 1 out
206 of 5 in the S2 group (Fig. 7E). Collectively, this shows that the chimaeric rIBVs are able to induce
207 a degree of local protection against the replication of IBV in the trachea.

208 To assess if the rIBVs induced humoral antibody responses following vaccination with BeauR-
209 M41(S), BeauR-M41(S1) and BeauR-M41(S2) viruses, IBV-specific serum titres were assessed
210 at 2 and 4 dpc. At 2 dpc, there was clear evidence of a boost in antibody titres in the BeauR-
211 M41(S) and BeauR-M41(S2) vaccinated groups (Fig. 8A), with significantly higher titres
212 compared to Mock/M41 controls ($P<0.001$). IBV induced antibody titres at 2 dpc in BeauR-
213 M41(S) vaccinated chickens were higher than those from BeauR-M41(S1) and BeauR-M41(S2)
214 vaccinated chickens across the dilution series (Fig. 8A). At 4 dpc, serum antibody titres from all
215 vaccinated groups were significantly higher compared to the Mock/M41 titres (Fig. 8B),
216 suggestive of a primed antibody response in the vaccinated chickens. The serum antibody titres at
217 14 dpc indicated no significant differences between the vaccinated groups and the challenge-only
218 controls (Fig. 8C), suggesting that a boosted response was lacking in response to challenge with
219 wild-type virus.

220 The virus neutralisation activity of the serum collected at 4 and 14 dpc were assessed and at 4 dpc
221 there was no neutralisation of the virus detected (data not shown). At 14 dpc, only serum from

222 BeauR-M41(S) and BeauR-M41(S1) vaccinated chickens had significantly higher neutralisation
223 activity of the virus compared to Mock/Mock control (*P*=0.002 and *P*=0.0066, respectively; Fig.
224 9A). BeauR-M41(S) vaccination induced significantly higher virus neutralisation titres compared
225 to BeauR-M41(S2) vaccination (*P*=0.04), whereas there was no significant difference in titres
226 compared with serum from BeauR-M41(S1) vaccinated or Mock/M41 challenge-only group (Fig.
227 9A). The levels of virus neutralisation activity detected were moderately positively correlated to
228 the anti-IBV serum titres (r^2= 0.5, *P*=0.002, Fig. 9B).

229 **Characterisation of rIBVs *in vitro*.** Following on from the observation of differences during the
230 *in vivo* vaccination trials, to elucidate whether the inclusion of a chimaeric S gene or a foreign S
231 gene had an effect on viral replication, the replication kinetics of rIBV BeauR-M41(S), BeauR-
232 M41(S1) and BeauR-M41(S2) viruses were investigated *in vitro*. At 12 hpi all viruses had similar
233 titres (Fig. 10A and 10B). This suggests that the inclusion of a foreign S gene, or a chimaeric S
234 gene has not impeded replication *in vitro* in either chicken kidney cells (CKCs) derived from Valo
235 chickens (Fig. 10A) nor CKCs derived from Rhode Island Red (RIR) birds (Fig. 10B). Single-
236 step growth curves performed in CKCs derived from RIR birds show that over the latent period
237 (2-8 hpi), BeauR-M41(S2) had lower virus titres compared to the other viruses, however when the
238 exponential growth was compared there was no statistical difference between the viruses (Fig.
239 10C). The titres of BeauR-M41(S) and BeauR-M41(S1) are similar over all time points (Fig. 10C).

240

241 **DISCUSSION**

242 We have previously shown that rIBVs expressing the ectodomain of the Spike protein of a
243 pathogenic strain in the context of an apathogenic strain BeauR, could induce increased levels of

protection against homologous and partially against heterologous challenge infection. Here, we extended this work and replaced only the S1 subunit of the ectodomain of BeauR with the S1 domain of M41 or QX, representing two strains that circulate in poultry flocks worldwide. These rIBVs have the advantage of being able to replicate in Vero cells, potentially allowing large scale vaccine production in cell culture rather than in embryonated eggs. In this first vaccination study, using a single dose of BeauR-M41(S1) or BeauR-QX(S1) in 1-week-old chicks, the birds were not protected against homologous challenge based on ciliary activity and clinical signs. Vaccination with BeauR-QX(S1) induced significantly higher serum titres post-challenge and the clinical signs associated with challenge virus, although present, decreased rapidly compared to unvaccinated birds challenged with QX. Together, these data show that vaccination with chimaeric rIBVs are able to induce a degree of virus-specific immunity with partial local protection in the mucosal head tissues and the primary site of replication, the trachea.

In attempt to address the questions of whether a full homologous S is required for optimal folding, virus replication and protection using an apathogenic recombinant virus, a second vaccination experiment was performed. One-week-old birds were immunised once with BeauR-M41(S), BeauR-M41(S1), or BeauR-M41(S2). Replacement of the apathogenic BeauR-S1 or S2 subunits with a S1 or S2 from a pathogenic strain, allowed BeauR to remain apathogenic, suggesting that the S1 or S2 alone do not play a role in the pathogenicity of IBV. This further expands our previous work showing that spike switching of BeauR-S with M41-S showed no effect on pathogenicity (23). Here, vaccination of chickens with a rIBV based on a BeauR backbone expressing a full S gene from the donor serotype enhanced the level of protection afforded against tracheal ciliostasis, with 3 out of 5 birds classed as fully protected. However, when classified under European Pharmacopeia standards for assessment of IBV vaccines (28), at which 80% protection (at a group

267 level) against ciliostasis is required, the BeauR-M41(S) vaccinated group was only able to confer
268 partial protection (~60%), and therefore is still not satisfactory for the criteria used for the
269 assessment of IBV vaccines for industrial application. Consistent with previously published work,
270 we show that collectively as a group the chickens vaccinated via ocular-nasal routes with BeauR-
271 M41(S) had ~60% ciliary activity remaining, a reduction in clinical signs and viral load post-
272 challenge (25). The protection seen at the trachea may potentially be improved with assessment of
273 ciliostasis at a later time-point, as Armesto *et al*. (23) reported that vaccination with BeauR-4/91(S)
274 gave ~60% ciliary activity at 4 dpc, which then improved to 90-100% at 6 dpc. In Trial 1, viral
275 RNA load in the trachea and CALT from the S1 vaccinated groups was reduced at 2 dpc, whereas
276 in Trial 2, all vaccinated groups had a clear significant reduction in viral RNA load at 2 and 4 dpc
277 in trachea and CALT. The qPCR used here is designed to detect the 5'UTR region of the genome
278 (29) and it therefore may be detecting incomplete virions or challenge virus captured in the lumen
279 of the trachea. To further support the viral RNA load data, infectious viral load recovered from the
280 trachea in both Trial 1 and 2 were lower in rIBV vaccinated chickens, indicating a degree of local
281 protection at the site of infection, which was not robust enough to completely protect against viral
282 replication *in vivo* and the loss of ciliary activity.

283 The major surface glycoprotein of coronaviruses, spike, is a type 1 glycoprotein and has two
284 structurally distinct conformations, pre-fusion and post-fusion (30-32). In the coronavirus
285 replication cycle the spike mediates the critical steps of receptor binding and membrane fusion.
286 Upon binding of the S1 receptor binding domain to the host cell, an irreversible conformational
287 switch to the post-fusion state allows the S2 subunit to fuse viral and cellular membranes,
288 facilitating entry of the viral genome and therefore downstream viral replication (32-34). Recently,
289 the crystal structure of the pre-fusion spike from Mouse hepatitis virus (MHV) and Human

290 coronavirus (HCoV HKU1) were resolved, highlighting the critical role that the interaction
291 between the trimers of S1 and S2 plays in stabilisation of the pre-fusion conformation of spike (31,
292 32). Here, expression of a chimaeric spike in a recombinant IBV backbone with the lack of a
293 homologous S2 possibly resulted in conformational changes either within the S1 subunit or
294 complete S protein, potentially affecting receptor binding and entry, but may have also altered
295 immunogenic epitopes. The S2 subunits of BeauR shares 87% and 97% amino acid sequence
296 similarity with QX and M41-CK, respectively, showing that there are only a few different amino
297 acid residues between them. The interactions between S1 and S2 sub-units are critical for
298 maintenance of conformation, recognition and efficient fusion of the spike to host cells; it has been
299 consistently shown that even a single amino acid change within the S2 subunit of coronavirus
300 spikes may influence the secondary structure of the overall spike or the S1 subunit (35, 36).

301 The development of a cryo-EM structure of IBV M41 spike, highlighting the evolutionary
302 difference between the pre-fusion spike structures of IBV compared to *betacoronaviruses* and
303 *alphacoronaviruses*, nonetheless indicates a high degree of structurally similarity to porcine
304 *deltacoronavirus* (37, 38). This structural model of pre-fusion IBV spike will significantly aid in
305 addressing the challenges over whether (i) expression of a chimaeric spike in a rIBV backbone
306 causes conformational changes either within the S1 subunit or complete S or (ii) it is vital that
307 homologous "matched" S1 and S2 and their interactions are required to maintain the correct pre-
308 fusion conformation of spike, as suggested in other coronaviruses.

309 The Beaudette strain, used here in the reverse genetics system, has an extended *in vitro* tropism,
310 ability to grow in cell cultures and an apathogenic nature, making it an excellent resource for
311 investigation of heterologous genes and growth characteristics of rIBV. During embryo passages
312 however the Beaudette strain may have acquired mutations which are likely to contribute to its

lack of pathogenicity and restrict its *in vivo* tropism and replication. Replacement of the BeauR S1 or S2 with corresponding subunits from a pathogenic strain did not indicate a significant impairment of *in vitro* growth of the viruses in comparison to the BeauR virus, showing no indication that BeauR-M41(S1) and BeauR-M41(S2) were unable to enter the cells, fuse with cell membranes or failed to replicate *in vitro*. Nevertheless, the lack of full protection afforded by BeauR rIBVs against wild-type challenge and the limited *in vivo* replication, strongly suggest that attenuations have occurred in genes playing an essential role in replication and these are negatively impacting upon its suitability as a vaccine vector. Development of an alternative, less attenuated backbone for expression of heterologous genes in rIBVs may promote the development of these live attenuated vaccines for the control of IBV.

Expression of the IBV S1 subunit alone has been shown to induce virus neutralising antibodies, albeit often requiring repeated vaccination (8, 10). Here, immunisation of chickens with rIBVs based on the Beaudette backbone expressing either M41 S or chimaeric S1/S2 induced virus neutralising antibodies, however the Mock/M41 serum also had a degree of neutralising activity. BeauR-M41(S) vaccinated chickens had significantly higher virus neutralising titres compared to BeauR-M41(S2) group but there was no statistical difference with the BeauR-M41(S1) group, showing that following a single vaccination with rIBV expressing M41(S1) neutralising antibodies are induced.

Live attenuated vaccines against IBV need to induce a good level of mucosal immunity with local tracheal and cell-mediated immunity also playing an important role in prevention of IBV infection (39, 40, 42). As discussed earlier, the BeauR backbone is impeded by poor *in vivo* replication and the lack of protection shown against ciliostasis indicates that there is a poor level of local immunity induced in the trachea by vaccination with BeauR rIBVs. Cytotoxic responses can also play a key

role in the early control of IBV as indicated by previous studies showing; NK cell activation (41), IBV-specific cytotoxic T-cell lymphocyte (CTL) activity of splenocytes isolated from IBV-infected chickens (42) and higher CTL proportions in respiratory tissues following IBV infection (43). Cellular infiltrates in the head-associated lymphoid tissues as well as a reduction in viral load in the trachea and CALT also implies that the rIBVs infected the chickens and suggests a possible role for the cell-mediated response. However, as we were unable to consistently detect the recombinant S1 viruses at 2 dpv, it raises possibilities that the viruses were either rapidly cleared from the tissues, replicate poorly at these sites of inoculation or have limited replication in a few cells which are below detectable limits of the assays. In the BeauR-M41(S) vaccinated group, over 50% of the chickens were positive for vaccine virus as assessed by RT-PCR, in the Harderian gland and nasal turbinates at 1 and 2 dpv, respectively. The primary site of IBV infection is thought to be the ciliated epithelium lining the trachea, however following ocular-nasal vaccination the virus has been detected in the nasal turbinates (44) and Harderian gland (45).

One possible explanation for poor protection of ciliary activity afforded by the recombinant S1 viruses could be that we only administered one single vaccine dose to the SPF chicks. Previous studies using baculovirus expressed IBV recombinant proteins or IBV purified proteins have required multiple injections to achieve a degree of protection in SPF chickens (10, 17). There is also evidence of an impaired humoral response in young chicks with regards to IBV vaccination; vaccination of 1-day and 7-day-old chicks showed a delay in both systemic and local IgA and IgG levels compared to vaccination of older chicks (14, 21 or 28-day-old) (46). Here, in an attempt to improve the protection against respiratory signs and ciliostasis with the recombinant S1 viruses, a prime/boost approach may aid in overcoming these potential issues.

In summary, we have previously generated recombinant IBV based on a BeauR backbone expressing a heterologous S1 from M41 or QX, and in the present study we have shown that a single vaccination in young chicks with these rIBVs although not adequate to completely prevent ciliostasis and clinical signs, they can induce a degree of virus-specific protective immunity. This was characterised by reduction in viral load recovered from trachea and CALT, cellular infiltrations at head mucosal and inoculation sites, higher serum anti-titres in vaccinated groups and induction of virus neutralising activity. Vaccination with BeauR-M41(S), despite expressing the homologous full S to attempt to overcome any issues with heterologous S1 and S2 subunits and suboptimal folding, only induce a partially protection against the loss of ciliary activity. As *in vitro* growth characteristics shows that inclusion of a foreign S gene or a chimaeric S gene in the rIBVs does not impede replication *in vitro* it suggests that the attenuated Beaudette backbone has hindered the *in vivo* replication of these rIBVs and to improve protection, multiple vaccinations or an alternative backbone may be required.

MATERIALS AND METHODS

Ethics statement. All animal experimental protocols were carried out in strict accordance with the UK Home Office guidelines and under licence granted for experiments involving regulated procedures on animals protected under the UK Animals (Scientific Procedures) Act 1986. The experiments were performed in The Pirbright Institute (TPI) Home Office licensed (X24684464) experimental animal house facilities and were approved by TPI animal welfare and ethical review committee under the terms of reference HO-ERP-01-1. Trial 1 used SPF Rhode Island Red (RIR) chickens obtained from TPI Poultry Production Unit in Compton. Trial 2 used the same chicken breed but obtained from The National Avian Research Facility in Edinburgh.

Cells and viruses. Tracheal organ cultures (TOCs) were prepared from 19-day-old SPF RIR chicken embryos (47-49). Primary Chicken Kidney (CK) cells were prepared by The Central Services Unit, TPI from kidneys extracted from either 2 to 3-week-old SPF RIR chickens or 2-week-old SPF derived Valo chickens (49). The pathogenic M41 strain (50) used in this study had previously been adapted in CK cells to produce M41-CK (Accession number X04722) (25). The pathogenic strain, QX (QX L1148 strain, Accession number KY933090) (51), was donated by Prof. Richard Jones, University of Liverpool. The rIBVs BeauR-M41(S), BeauR-M41(S1), BeauR-M41(S2) and BeauR-QX(S1) used herein are described in a schematic illustration (Fig. 1) and constructed using the backbone of Beau-R, which is the molecular clone of Beau-CK (Accession number AJ311317) (21, 26). All isolates of IBV and rIBV were propagated in 10-day-old RIR SPF embryonated eggs. Allantoic fluid was clarified by low speed centrifugation, 24 to 48 hours post infection (hpi). Titrations to determine virus infectivity were either performed in TOCs as described by (25), or in CK cells (49); titres are expressed as 50% (median) ciliostatic doses (CD50) per ml or plaque forming unit (PFU) per ml, respectively.

Analysis of growth kinetics in CK cells. Confluent CK cells seeded in either 6-well or 12-well plates were inoculated with 10^4 PFU rIBV or IBV for multi-step growth curves or 10^5 PFU rIBV or IBV for single-step growth curves in 0.5 ml serum-free N,N-Bis(2-hydroxyethyl)-2aminoethanesulphonic acid (BES) medium and incubated for 1 h at 37°C, 5 % CO_2. Cells were washed with phosphate buffered saline a (PBSa) to remove residual virus and 2 ml of serum-free BES medium was added per well. Extracellular virus was harvested at defined intervals and assayed by titration in CK cells.

Experimental design of *in vivo* vaccination/challenge trials. SPF RIR chickens were housed in positive-pressure, HEPA-filtered isolation rooms in which each group was housed in a separate

404 room. In two separate experiments, birds were randomly divided into 5 groups of 30 birds for Trial
405 1 and 5 groups of 40 birds for Trial 2. Eight-day-old chicks were inoculated (classified as primary
406 inoculation) with 10^5 PFU of BeauR-M41(S1) or BeauR-QX(S1) (Trial 1) or 10^4 PFU BeauR-
407 M41(S), BeauR-M41(S1) or BeauR-M41(S2) (Trial 2) in a total of 0.1 ml of PBS via conjunctival
408 (eye drop) and intranasal routes. A challenge dose, equal to the primary inoculation, 10^5 PFU
409 (Trial 1) and 10^4 PFU (Trial 2) of the corresponding wild-type viruses were administered in the
410 same manner 21 days after the primary inoculation to the appropriate groups. Of note, the IBV QX
411 strain used here could not be propagated in CK cells, so a CD_{50} dose of $10^{2.73}$ was used. Mock-
412 infected controls were inoculated via the same route with 0.1 ml of PBS and mock/challenge
413 control groups were inoculated with 0.1 ml PBS and challenged with the same dose of wild-type
414 virus. Birds were euthanised by cervical dislocation at specific times post-infection and a panel of
415 tissues sampled to allow for downstream analysis. Blood samples were collected and processed
416 for the collection of serum. Clinical signs used to determine pathogenicity were snicking, rales and
417 ciliary activity of the trachea (a bird was considered protected if 50% or more ciliary activity was
418 retained in 9 out of 10 tracheal rings, this must be in 80% of the group) (28, 52).

419 **Isolation of tissues: Virus isolation and ciliostasis assay.** Tissues collected were divided into
420 two parts; one part was stabilised in RNAlater® (Ambion) for RNA extraction and the other in
421 20% sucrose/PBS (0.22µM filtered) at 4ºC overnight before snap freezing in OCT (Thermo
422 Scientific) for histology. Tissues collected included: Harderian gland, CALT, NALT and trachea.
423 Tissues were removed at 2 and 4 days post-vaccination (dpv), and at 2, 4 and 14 dpc. Tracheas
424 were removed from five randomly selected chickens from each group at 4 dpv and 4 dpc for
425 assessment of ciliary activity as described previously (25). Part of the trachea and CALT tissues
426 were stored in PBS for virus isolation.

427 **Detection of viral RNA.** For virus isolation and RNA extraction, tissues stored in PBS and RNA-
428 later, respectively, were freeze-thawed and homogenised using the TissueLyser II (Qiagen), as
429 described in (23). Total RNA was isolated using the RNeasy® Mini Kit and DNase treated
430 following manufacturer's instructions (Qiagen). cDNA was synthesised from 1µg of tRNA using
431 Superscript IV Reverse Transcriptase (Life Technologies) with a random oligo primer as per
432 manufacturer's instructions. To quantify infectious viral load in trachea, tissue derived supernatant
433 was titrated in TOCs. To determine whether infectious virus was present, 10-day-old SPF
434 embryonated eggs were inoculated with 100 µl Allantoic fluid, at 24 – 48 hpi they were assessed
435 for viral presence by RT-PCR using primers specific for the 3'UTR, as described by (53). For
436 quantification of viral load, qPCR was performed using the Taqman Universal PCR Master Mix
437 (Applied Biosystems) with primers and probes specific to the 5' UTR region, as described by (29).
438 Serial dilutions of M41 cDNA (generated from 1µg tRNA) were included to generate a standard
439 curve and data expressed in terms of the cycle threshold (Ct) value, were normalised using the Ct
440 value of 28S cDNA product for the same sample (54).

441 **Infectious Bronchitis Virus ELISA.** Serum samples collected at 21 dpv (pre-challenge), 2, 4 and
442 14 dpc were assayed with the commercial IDEXX IBV antibody test kit (IDEXX laboratories). To
443 determine the end-point titre the serum samples were two-fold serially diluted in the range 1:20 –
444 1:2560 prior to incubation. After sample incubation, the remaining steps were followed directly
445 according to the manufacturer's instructions. The sample/positive (S/P) ratio was determined by
446 the following equation = (Mean sample – Mean Kit Negative)/(Mean Kit positive – Mean Kit
447 Negative). S/P ratios above 0.2 were considered to be positive for IBV antibodies. Polyclonal
448 chicken serum raised against M41 and QX serum were included on each independent test plate
449 (GD Animal Health).

Immunocytochemistry. For fluorescent microscopy, cryostat sections (5µm) were fixed in acetone, washed in PBS, and blocked for 1 h at RT with 10% normal goat serum and 0.5% bovine serum albumin in PBS (blocking buffer). Slides were washed and incubated for 1 h with optimally diluted primary antibodies (anti-Bu-1 (clone AV-20, AbD Serotec), anti-CD8α (clone 3-298, AbD Serotec); anti-CD8β (clone EP42, AbD Serotec) and anti-CSF1R (55) or isotype controls, all diluted in blocking buffer. Sections were washed and incubated with an Alexa Fluor 488-labeled goat anti-mouse IgG_1/IgG_{2a} or Alexa Fluor 568-labeled goat anti-mouse IgG_1/IgG_{2b} according to the appropriate isotype, diluted in blocking buffer for 1 h. Nuclei were visualized using DAPI (Invitrogen). Images were captured with a Leica DMLB fluorescence microscope with a coupled device digital camera and analysed using ImageJ analysis software. For light microscopy, cryostat sections (5µm) were fixed in acetone and stained with Harris' Haematoxylin (Sigma-Aldrich) and 1% Eosin (Sigma-Aldrich). Sections were dehydrated through graded ethanols and xylene and mounted in a xylene-based medium (DePex, Gurr-BDH Chemicals). Images were captured with a Hamamatsu Nano-zoomer-XR digital slide scanner.

Analysis of neutralising antibody. Virus neutralisation tests were performed by GD Animal Health (56). Briefly, two-fold serial dilutions of serum were made in a 1:1 mixture of Medium-199 and Ham's F10 in 96-well plates. To each well an equal volume of CEK cells (in medium supplemented with 10% FCS) were added. After culture with M41 for 3-4 days at 37ºC with 5% CO_2, cell monolayers were examined for CPE. All individual titres were expressed as $\log_2$ of the reciprocal of the highest serum dilution that showed complete inhibition of CPE.

Statistical analyses. Viral load qPCR data were tested for normality through residual plots and the difference between the mean corrected 40-Ct values were statistically evaluated by the parametric one-way ANOVA test adjusted for post-hoc analysis, Tukey's pairwise comparison.

Serum antibody levels, viral isolation titres, ciliary activity and virus neutralisation titres were tested for normality and non-parametric analyses conducted. Differences between the groups were statistically evaluated by the non-parametric Kruskal-Wallis test adjusted for post-hoc analysis, Mann Whitney U pairwise comparison. The relationship between anti-IBV serum and virus neutralisation titres were compared by Spearman rank correlation analysis. Analysis of the viral growth curves was conducted by fitting a polynomial curve to the exponential phase of viral growth (57), growth rates were then compared between groups by the non-parametric Kruskal-Wallis test adjusted for post hoc analysis. For all statistical analyses, *P* values of less than 0.05 were considered significant. All statistical analysis was conducted in MiniTab version 17 or GraphPad Prism 7.

ACKNOWLEDGEMENTS

This project was funded by the BBSRC Animal Research Club with grant numbers BB/M012784/1 and BB/M012069/1. This work was additionally supported by the Institute Strategic Programme Grant funding from the BBSRC to The Roslin Institute with the grant number BB/J004324/1.

We would like to thank all animal services staff at The Pirbright Institute (TPI) for their excellent assistance in running the animal experiments, and all members of the Coronavirus group at TPI and Dr. Dominika Borowska for their help with collecting and processing samples during the animal experiments.

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659 **Figure legends**

660 **Figure 1.** Design of rIBV constructs**.** Schematic of wild type BeauR and rIBV genomes generated
661 by reverse genetics to display homologous spike genes in Beaudette backbone. The rIBVs
662 generated expressed either the S1 and/or S2 ectodomain and transmembrane domain (TM) from
663 M41 and QX wild-type virus; with M41 derived genes represented by red boxes and QX derived
664 genes represented by green boxes. In all rIBVs the Beaudette backbone is represented by solid blue
665 boxes and the endodomain (E) of S2 from Beaudette is represented by shaded blue boxes. *BeauR-
666 M41(S) displays the full ectodomain of M41 spike, as previously described (22).

667

668 **Figure 2.** Assessment of clinical signs associated with BeauR-M41(S1) and BeauR-QX(S1)
669 infected chickens following challenge with M41-CK or QX. (A) Snicking and (B) Rales (n=10-
670 20 per group).

671

672 **Figure 3.** Cellular infiltrates of head associated lymphoid tissues following vaccination with
673 BeauR-M41(S1) and BeauR-QX(S1). (A and B) Harderian gland at 2dpv (C-E) CALT tissue at
674 2dpv. (A and C) Cryosections were stained with monoclonal antibodies to detect CSF-1R^{+} (red)
675 and CD8β^{+} (green) cells or (B and D) to detect Bu-1^{+} (green) and CD8α^{+} (red) cells.Nuclei were
676 labelled with DAPI (blue). The scale bars represent 50 µm. (E) H and E stained cryosections of
677 the lower conjunctiva, inset images depict CALT regions detected in (A) BeauR-M41(S), (B)
678 BeauR-QX(S1) tissues which were not clearly evident in (C) Mock lower conjunctivas. The scale
679 bars represent 250 µm. Representative images are shown for all.

680

681 **Figure 4.** Viral load in CALT and Trachea in BeauR-M41(S1) and BeauR-QX(S1) vaccinated
682 chickens following challenge with M41-CK or QX. (A-D). Relative viral RNA load (expressed as
683 corrected 40-Ct) at specific time-points: (A and C) 2 dpc, (B and D) 4 dpc. (E) Infectious viral load
684 titres in trachea at 4 dpc. Data points are shown as individual animals and lines represent mean and
685 standard error of mean (SEM). Statistically significant differences between groups are highlighted;
686 *, P <0.05; **, P < 0.01.

687

688 **Figure 5.** Measurement of serum anti-IBV titres of BeauR-M41(S1) and BeauR-QX(S1)
689 vaccinated groups. Serum titres were assessed by commercial ELISA at (A) 2 dpc, (B) 4 dpc, (C)
690 M41 groups at 14 dpc and (D) QX groups at 14 dpc. Pre-challenge titres (i.e. 21dpv) are included
691 in (C) and (D). The mean S/P (±SD) from each group (n=5-10) and includes four technical
692 replicates/animal. Dashed line shows the cut-off for positive samples (S/P=0.2). Solid bars denote
693 a trend in statistical significance across dilutions in comparisons with Mock/challenge only group
694 e.g. BeauR-QX(S1)/QX compared to Mock/QX and BeauR-M41(S1)/M41 compared to
695 Mock/M41; *, P<0.05.

696

697 **Figure 6.** Assessment of clinical signs associated with BeauR-M41(S), BeauR-M41(S1) and
698 BeauR-M41(S2) vaccination following challenge with M41-CK. (A) Snicking and (B) rales
699 (n=10-20 per group).

700

701 **Figure 7.** Viral load in CALT and trachea in BeauR-M41(S), BeauR-M41(S1) and BeauR-
702 M41(S2) vaccinated chickens following challenge with M41-CK. (A-D) Relative viral RNA load
703 (expressed as corrected 40-Ct) at (A and C) 2 dpc and (B and D) 4 dpc. (E) Infectious viral load

titres in trachea (4 dpc). Data points are shown as individual animals and lines represent mean (±SEM). Statistically significant differences between groups are highlighted; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 8. Measurement of serum anti-IBV titres of BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccinated groups. Serum titres were assessed by commercial ELISA at (A) 2 dpc, (B) 4 dpc and (C) 14 dpc. The mean S/P ratio (±SEM) from each group (n=10) includes four technical replicates/animal. Dashed line shows the cut-off for positive samples (S/P=0.2). Solid bars denote a trend in statistical significance across dilutions in comparisons with Mock/challenge only group e.g. BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) compared to Mock/M41; *, $P<0.05$.

Figure 9. Measurement of virus neutralisation antibody titres of BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccinated and Mock groups at 14 dpc. (A) Virus neutralisation titres were determined by titration of serum in CK cells. Virus neutralisation titres expressed as $\log_2$ of the reciprocal of the highest serum dilution that showed complete inhibition of CPE (n=5 or 10). Lines represent mean (±SEM). Statistically significant differences between groups are highlighted; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). (B) Relationship between virus neutralisation activity and 14 dpc anti-IBV serum titres. Data points represent the S/P ratios from individual serum samples (n=39) plotted against virus neutralisation titres, compared by Spearman rank correlation analysis.

Figure 10. Comparison of the growth curves of BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2). (A) Multi-step growth curve in chicken kidney (CK) cells derived from Valo chickens,

726 (B) 24 h growth curve and (C) single-step 12 h growth curves in CK cells derived from RIR
727 chickens. Supernatant was harvested at various time-points post-infection and titres of progeny
728 virus were determined by a plaque titration assay on CK cells. Data points represent mean of three
729 independent experiments and error bars represent SEM.

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Table 1. Assessment of protection against ciliostasis associated with BeauR-M41(S1) and BeauR-QX(S1) vaccination following challenge with M41-CK or QX.

Vaccination/Challenge	Mean ciliary activity (±SD)[1]	Number of birds with 90% ciliary activity[2]	Percentage of group protected[3]
Mock/Mock	92% (±8.2%)	5/5	N/A
Mock/M41	2% (±1.4%)	0/5	0%
BeauR-M41(S1)/M41	9% (±16.3%)	0/5	0%
Mock/QX	1% (±0%)	0/5	0%
BeauR-QX(S1)/QX	1% (±1.4%)	0/5	0%

[1]Mean ciliary activity per group calculated from ciliostasis scores for 10 tracheal rings per individual bird using formula = ((total ciliostasis score of tracheal rings)/40)*100.

[2]Ciliary activity assessed according to European Pharmacopeia standards (27) where bird is deemed protected against ciliostasis if no fewer than 9 out of 10 tracheal rings per bird showed normal ciliary activity (>50% ciliary activity retained).

[3]The vaccine is considered to be efficacious at conferring protection against ciliostasis when 80% or more of the birds in a group were protected.

Table 2. Assessment of protection against ciliostasis associated with BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2) vaccination following challenge with M41-CK.

Vaccination/Challenge	Mean ciliary activity (±SD)[1]	Number of birds with 90% ciliary activity[2]	Percentage of group protected[3]
Mock/Mock	96% (±5.2%)	5/5	N/A
Mock/M41	0% (±0%)	0/5	0%
BeauR-M41(S)/M41	65% (±36.2%)	3/5	60%
BeauR-M41(S1)/M41	19% (±33%)	1/5	20%
BeauR-M41(S2)/M41	23% (±43.4%)	1/5	20%

[1]Mean ciliary activity per group calculated from ciliostasis scores for 10 tracheal rings per individual bird using formula = ((total ciliostasis score of tracheal rings)/40)*100.

[2]Ciliary activity assessed according to European Pharmacopeia standards (27) where bird is deemed protected against ciliostasis if no fewer than 9 out of 10 tracheal rings per bird showed normal ciliary activity (>50% ciliary activity retained).

[3]The vaccine is considered to be efficacious at conferring protection against ciliostasis when 80% or more of the birds in a group were protected.

Table 3. Detection of IBV-derived RNA by RT-PCR in head associated lymphoid tissues and trachea samples following vaccination with BeauR-M41(S), BeauR-M41(S1) and BeauR-M41(S2).

Vaccination	Days post-vaccination	Number of virus positive tissues per group		
		Harderian gland	Trachea	Nasal turbinates
Mock	1	0/5	0/5	N/A
BeauR-M41(S)		3/5	0/5	N/A
BeauR-M41(S1)		1/5	0/5	N/A
BeauR-M41(S2)		1/5	2/5	N/A
Mock	2	0/5	0/5	0/5
BeauR-M41(S)		1/5	0/5	3/5
BeauR-M41(S1)		0/5	0/5	3/5
BeauR-M41(S2)		0/5	0/5	2/5
Mock	4	0/5	0/5	0/5
BeauR-M41(S)		3/5	0/5	2/5
BeauR-M41(S1)		1/5	0/5	2/5
BeauR-M41(S2)		1/5	2/5	3/5

The results are depicted as "number of positive samples/number of birds per group" (total of 5 birds/group). All positive results were confirmed by sequencing of PCR products (data not shown).

Fig 1

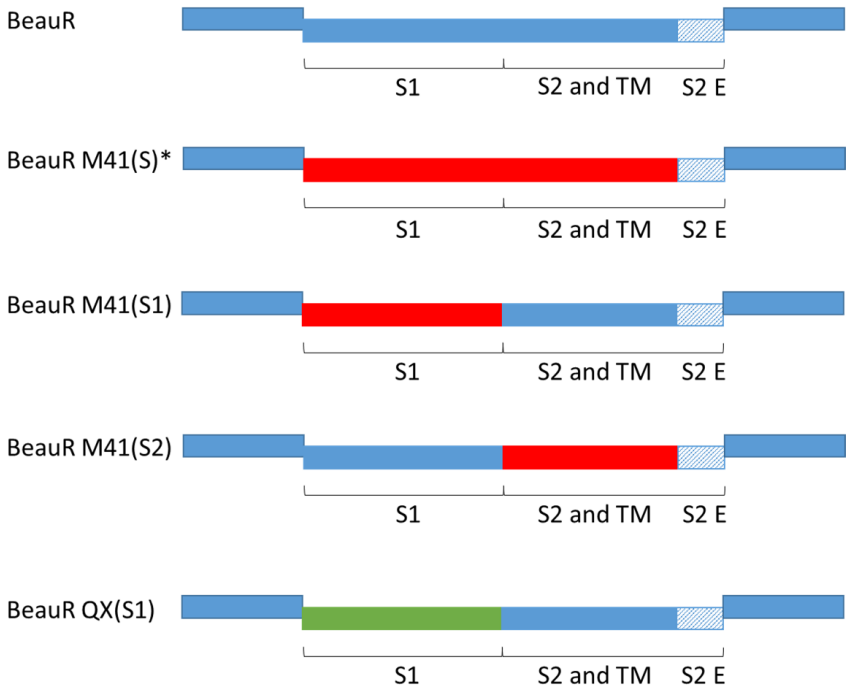


Fig 2

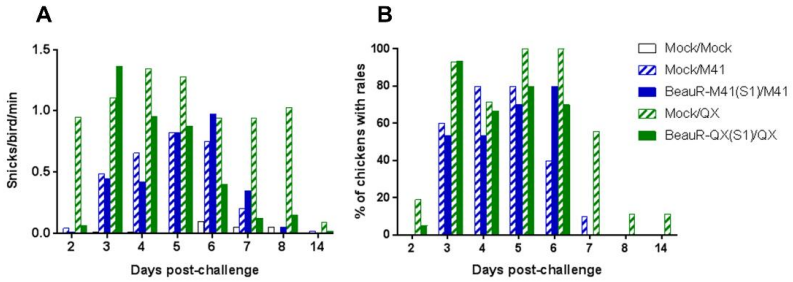


Fig 3

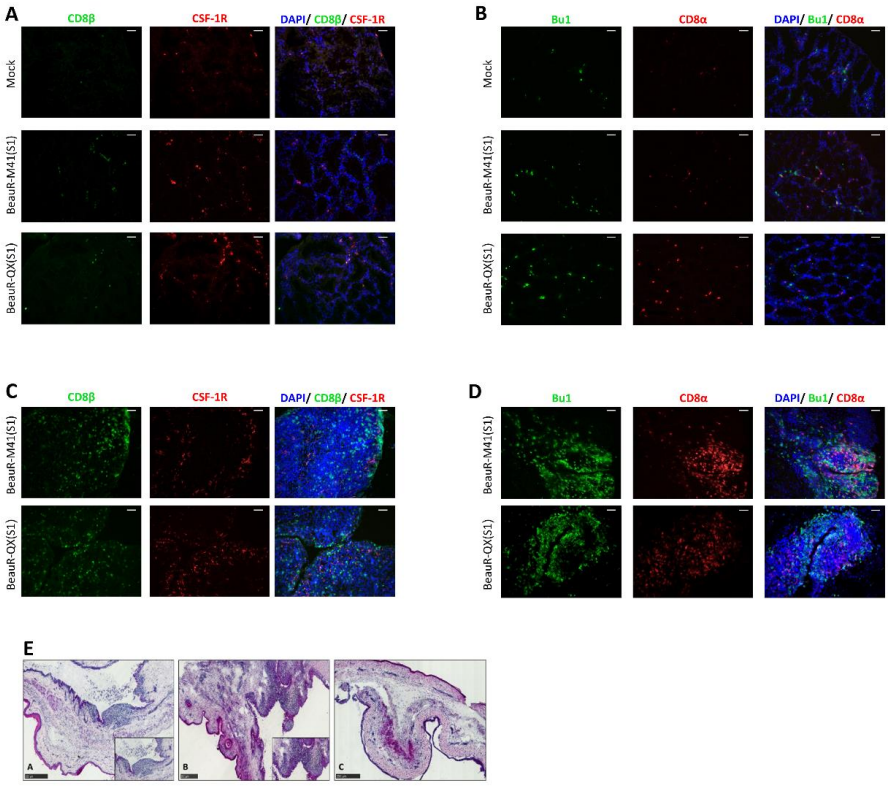
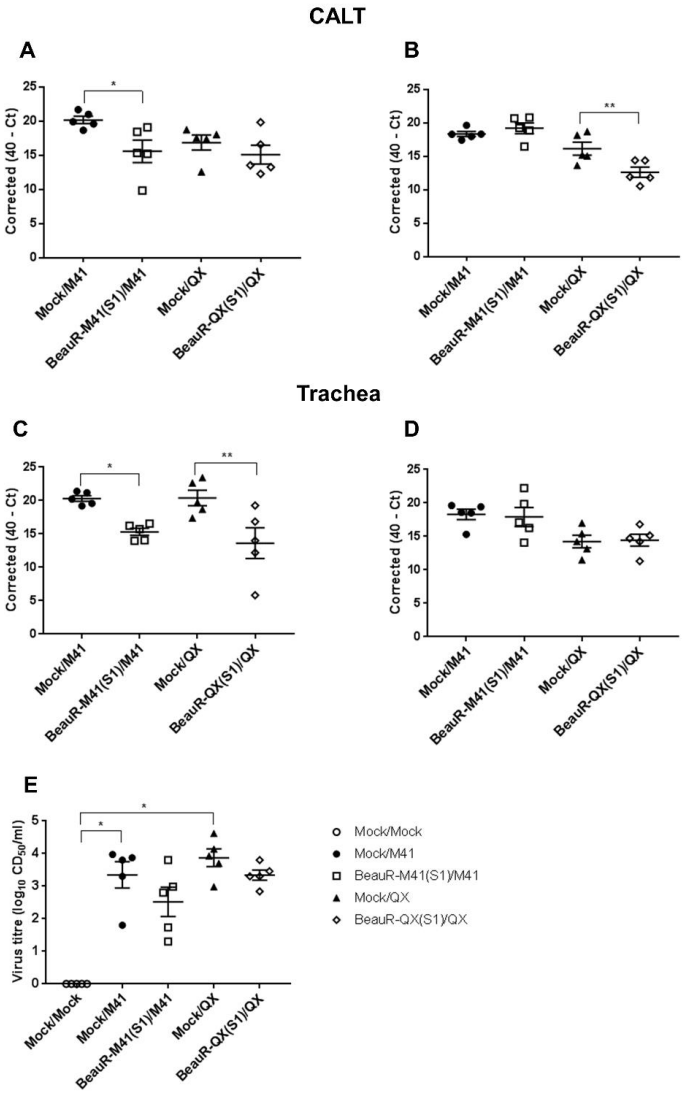


Fig 4



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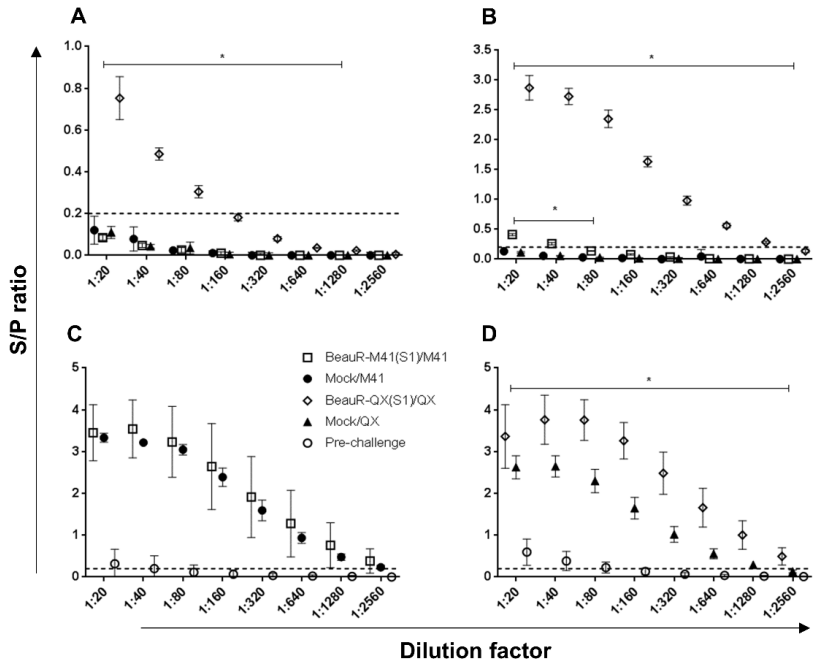

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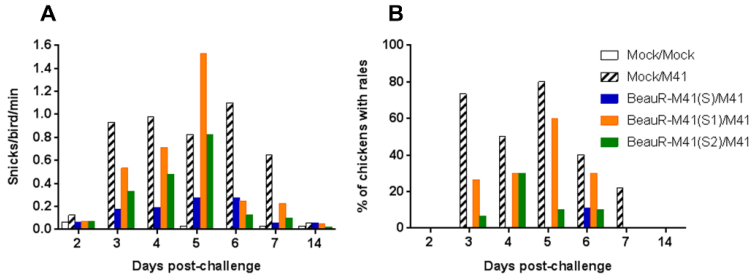

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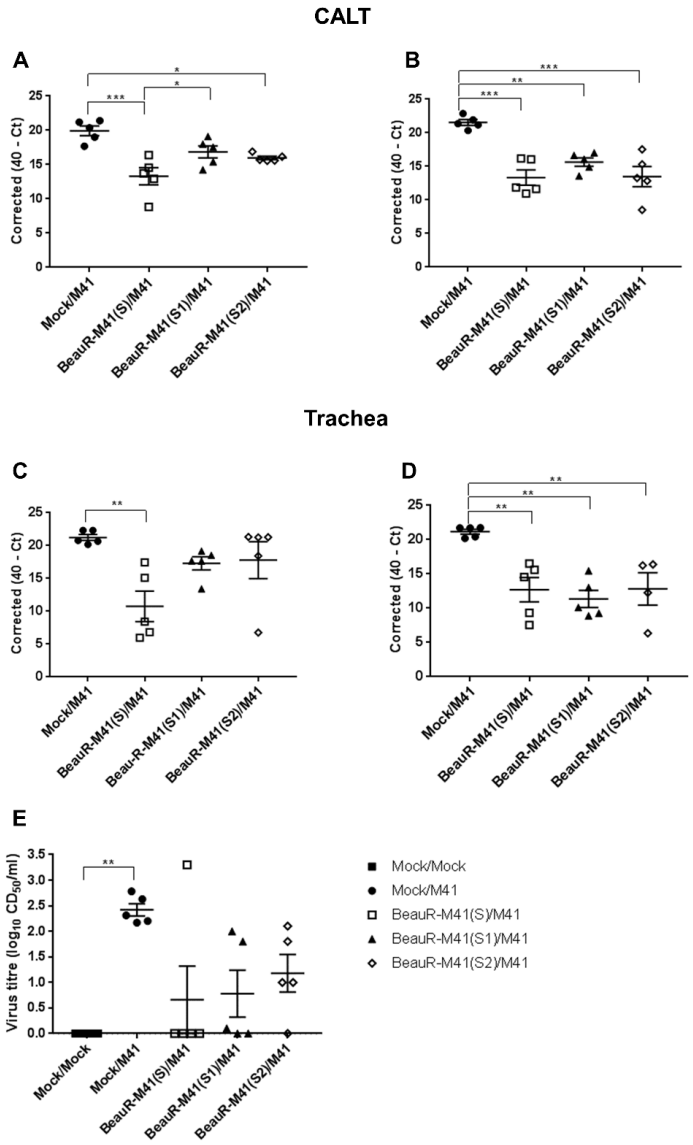

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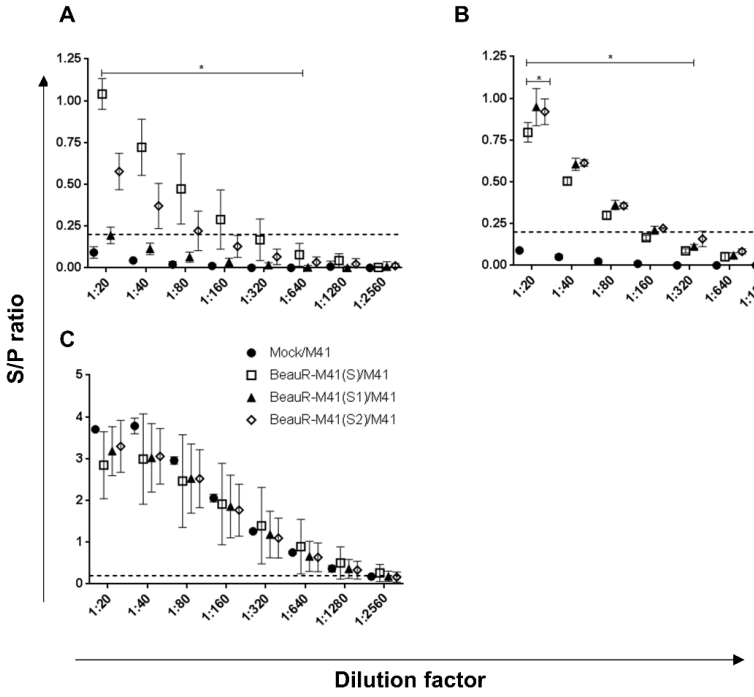

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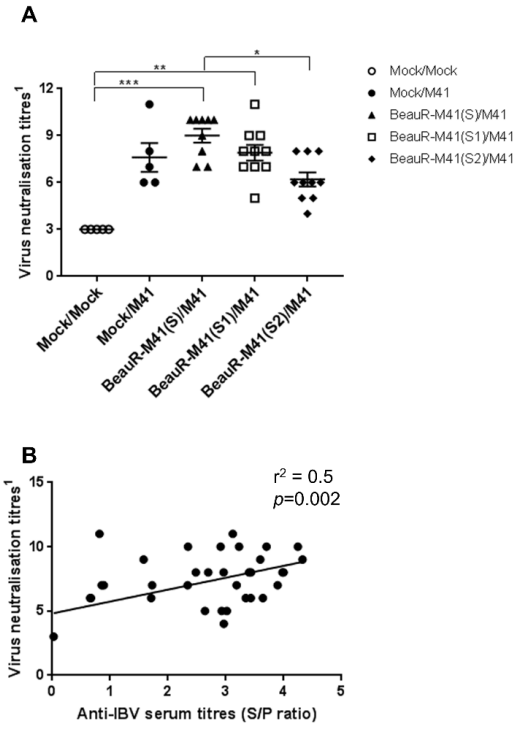

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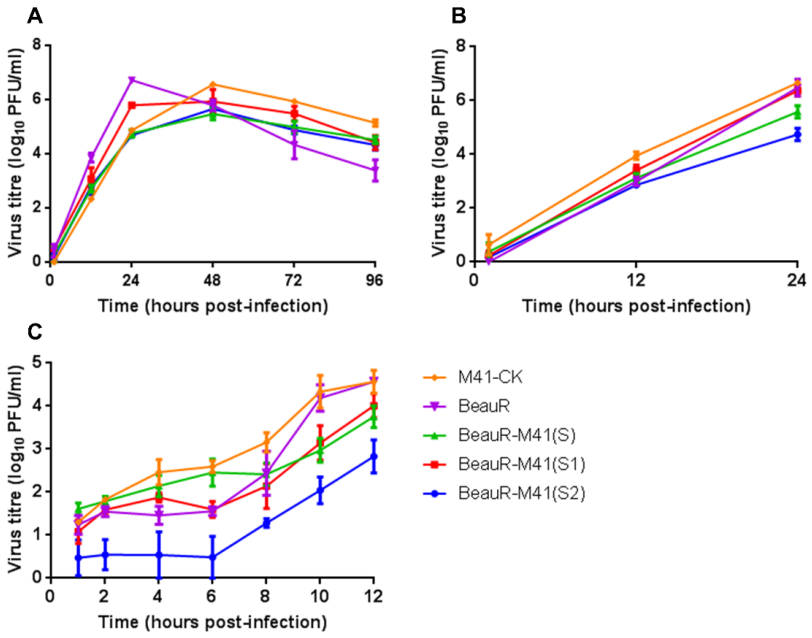
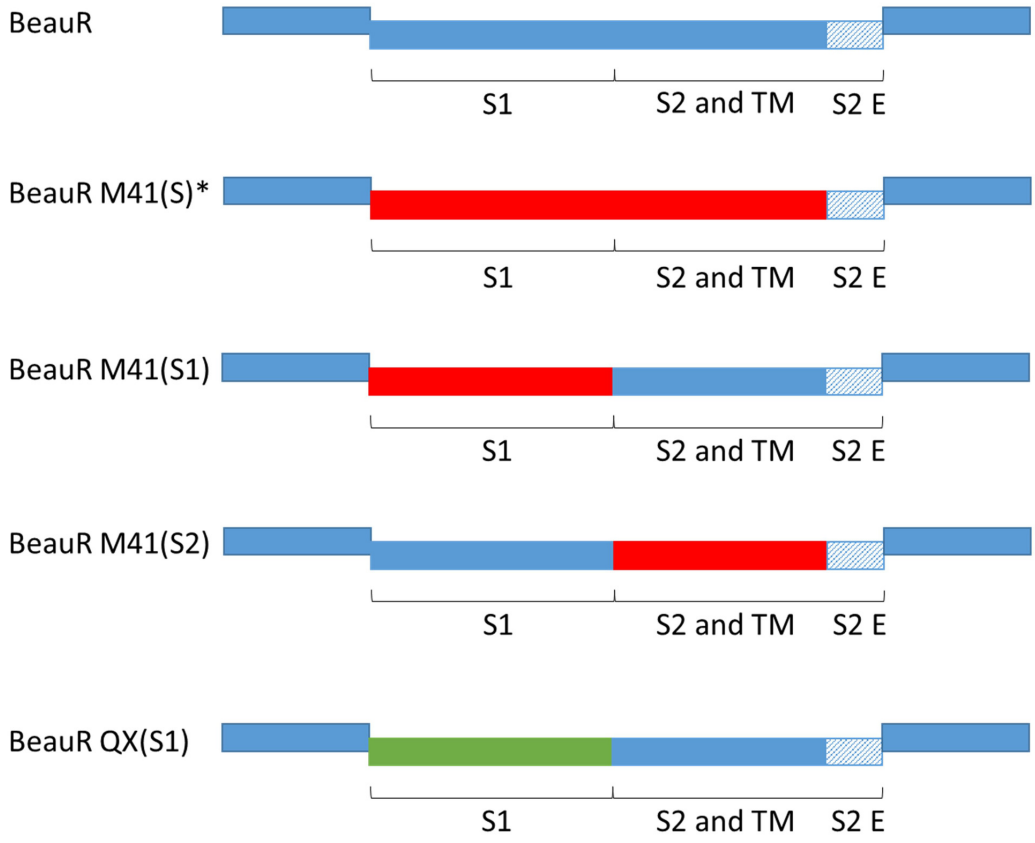


Fig 1



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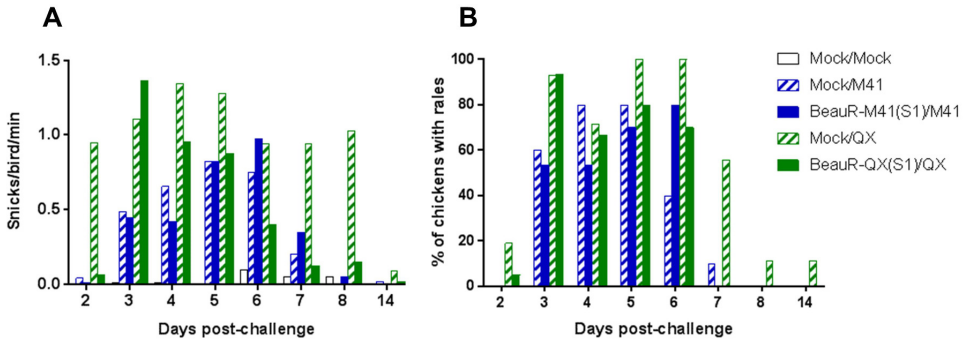

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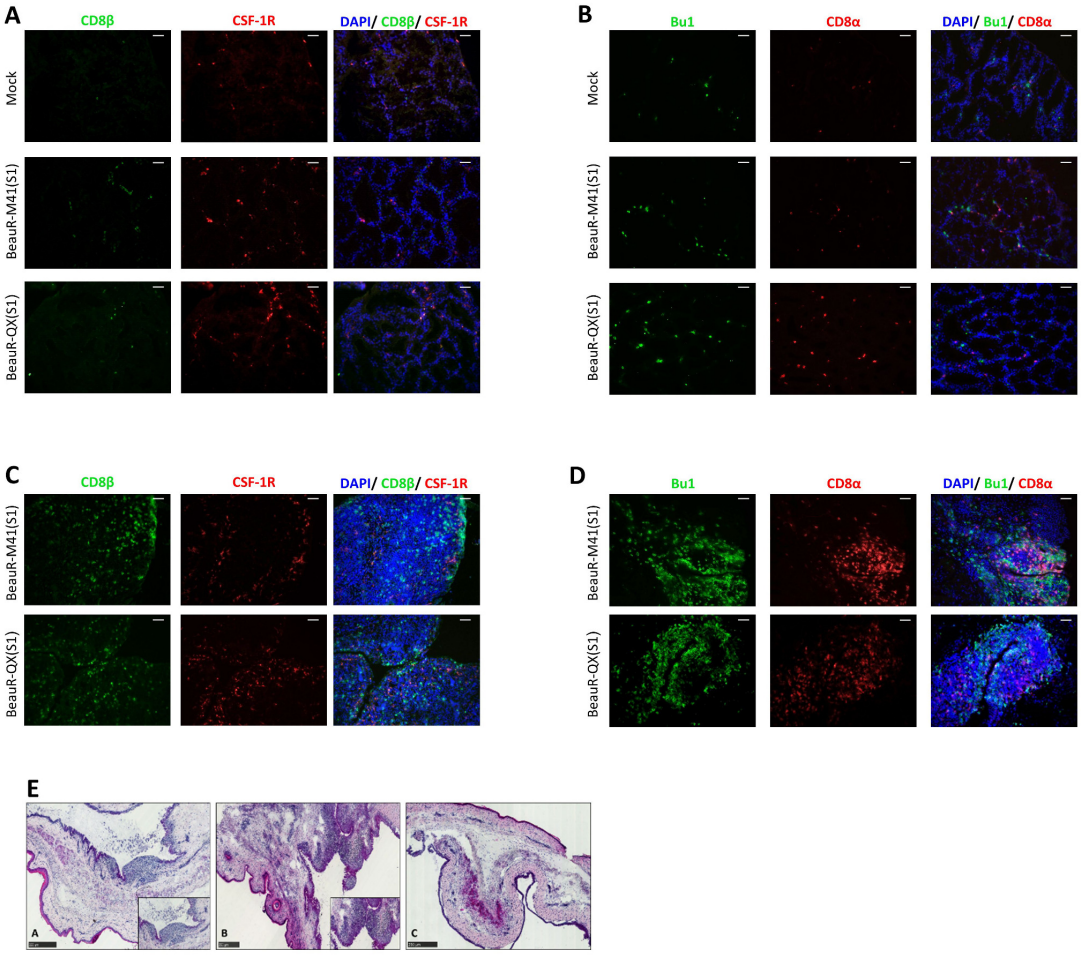

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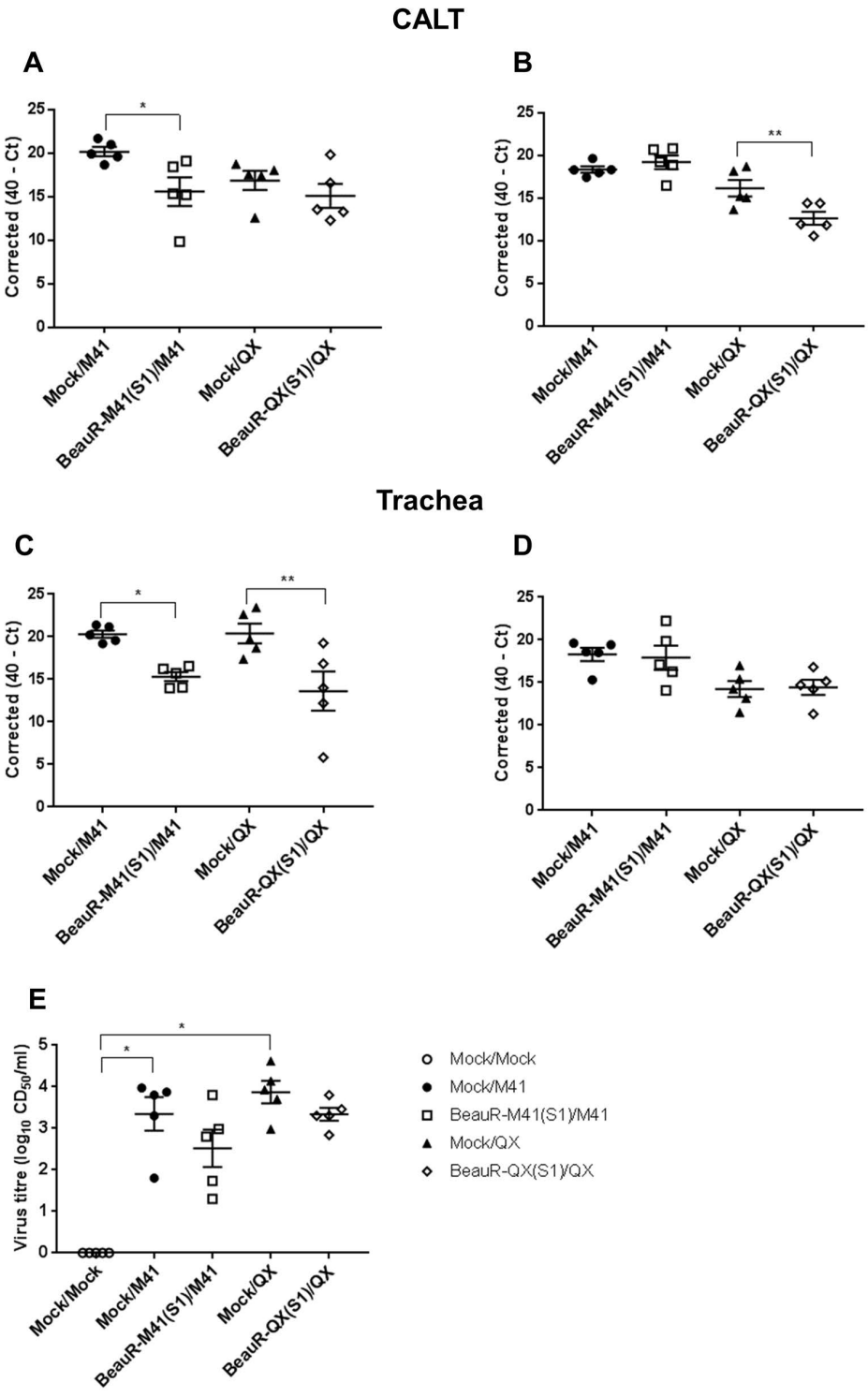

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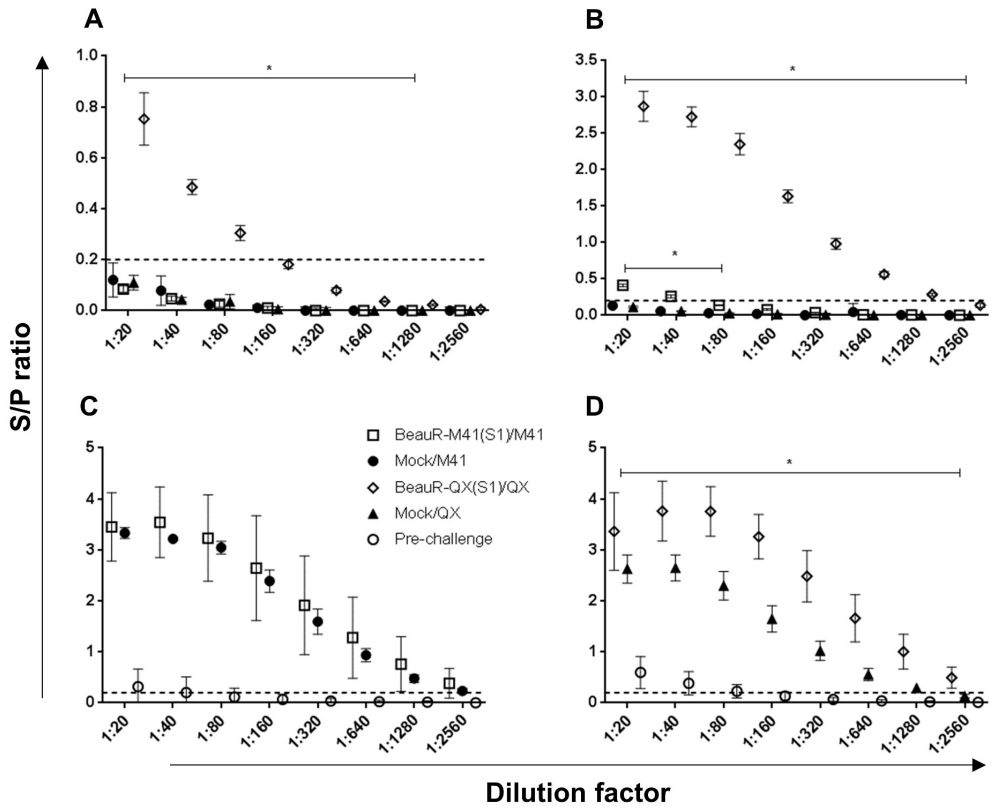

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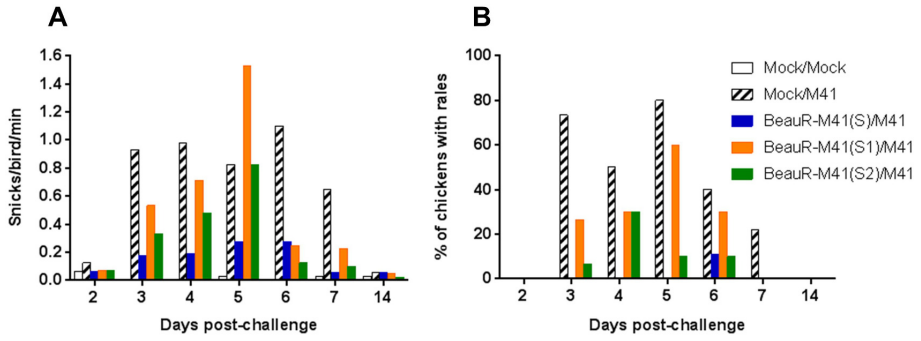

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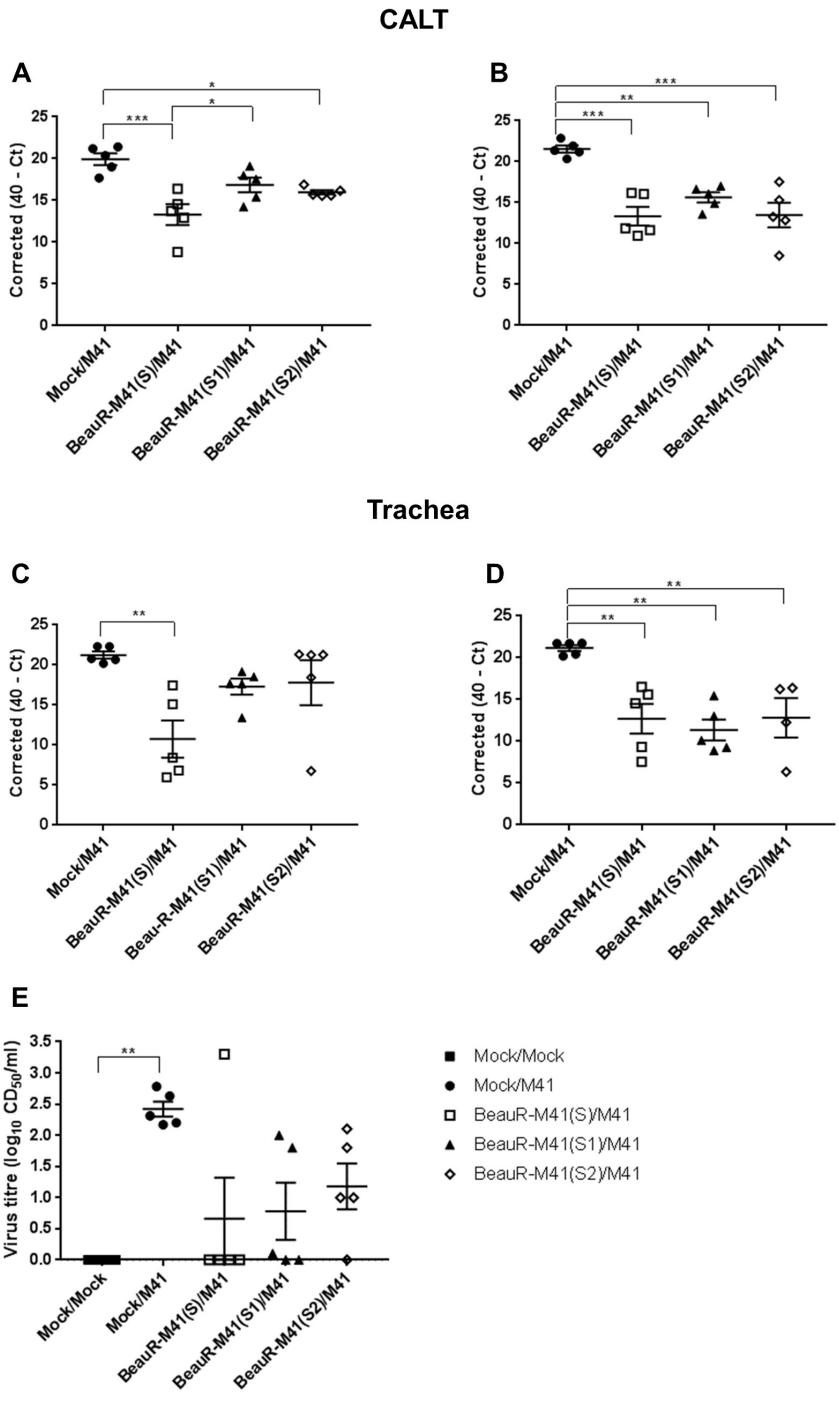

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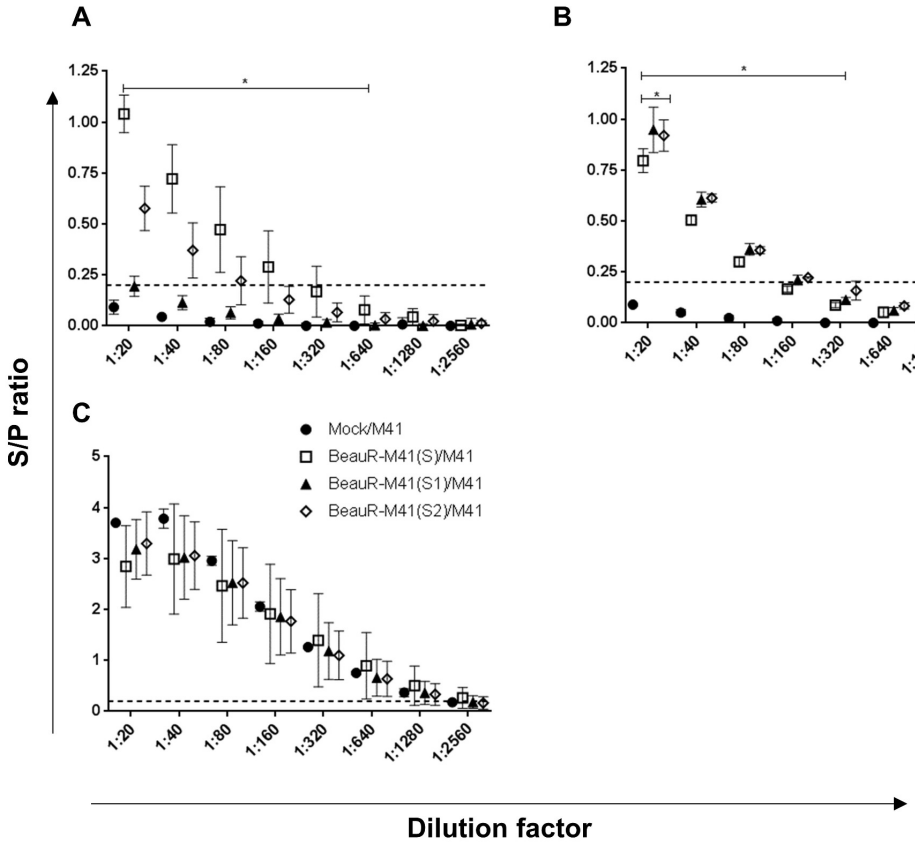

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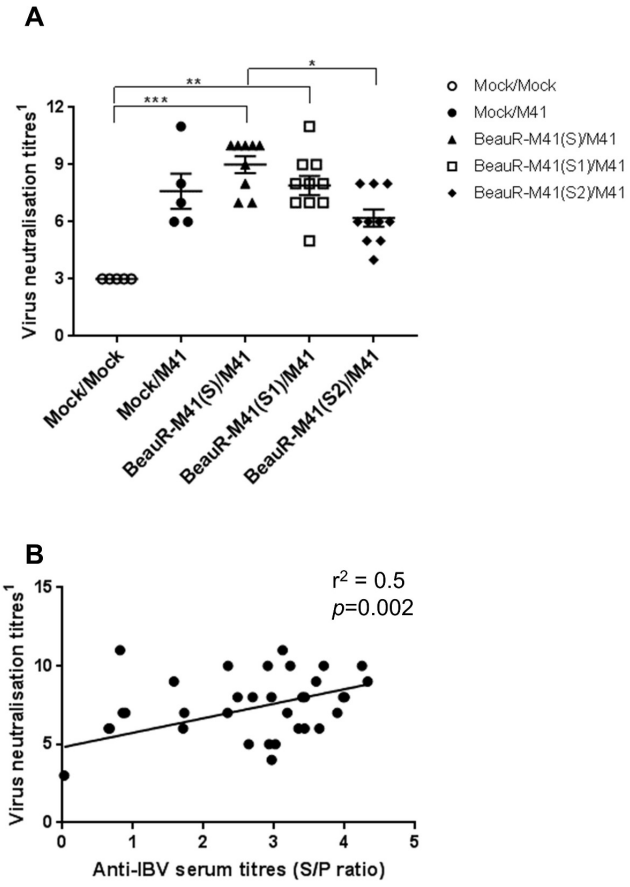

Fig 10